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Photoreactivation of Ultraviolet-Irradiated, Plasmid-bearing and Plasmid-free Strains of *Bacillus anthracis*

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Running Title: Photoreactivation of *Bacillus anthracis*

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ABSTRACT

The effects of toxin- and capsule-encoding plasmids on the kinetics of UV inactivation of various strains of Bacillus anthracis were investigated. Plasmids pXO1 and pXO2 had no effect on bacterial UV sensitivity or photoreactivation. Vegetative cells were capable of photoreactivation, but photo-induced repair of UV damage was absent in B. anthracis Sterne spores.

INTRODUCTION

Anthrax, which is caused by the toxigenic, spore-forming, gram-positive bacterium Bacillus anthracis, is a virulent and highly contagious disease to which most warm-blooded animals, including man, are susceptible. Virulent strains of B. anthracis produce a capsule composed of poly-D-glutamic acid and an exotoxin. The toxin is composed of three proteins identified as edema factor (EF), protective antigen (PA), and lethal factor (LF) (17). Anthrax toxin and capsule production are associated with two separate plasmids, pXO1 and pXO2 (5, 33, 35). Alum-precipitated antigenic material from culture filtrates containing PA is used as the current anthrax vaccine for humans, while live spores of the avirulent, non-encapsulated Sterne strain are used as a veterinary vaccine (7). Anthrax spores are extremely stable in a dry environment. The spores vegetate in the soil when the pH and temperature conditions are favorable and an organism-spore-organism cycle can be maintained for years (34).

Irradiation of cells with UV light (220-300 nm) can result in the formation of pyrimidine dimers in the DNA, leading to mutagenic changes or cell death. Several repair pathways exist for the repair of UV-induced DNA damage including photoreactivation, excision repair, recombinational repair, and inducible error-prone repair (15). Photoreactivation is the error-free, light-dependent (300-500 nm), enzymatic monomerization of UV-induced pyrimidine dimers. The photoreactivating enzyme has been found in many species of bacteria, (10, 13) as well as in plant and animal cells, including

human cells (31). Light-induced recovery from UV damage has been reported in vegetative cells of Bacillus mycoides, B. pumilis, and in some strains of B. megaterium and B. cereus, but not in B. polymyxa, B. circulans, and transformable B. subtilis (14, 28-29). DNA repair by photoreactivation in B. anthracis has not been reported previously. This study was conducted to determine whether or not B. anthracis can repair its UV-damaged DNA by photoreactivation. Studies were also made to determine what effects the two plasmids, which encode capsule and toxin production, and sporulation have on UV sensitivity and photoreactivation.

MATERIALS AND METHODS

Bacterial strains. B. anthracis Vollum 1B from the USAMRIID culture collection is a virulent (cap^+ tox^+) strain containing plasmids pXO1 and pXO2. B. anthracis Sterne from the USAMRIID culture collection is an avirulent (cap^- tox^+) veterinary vaccine strain which contains the plasmid XO1. Plasmid-free isolates (GK8N and GK40N) were generated by treating Sterne cultures with novobiocin. E. coli B/r (ATCC #12407), E. coli B_{s-1} hcr^- (ATCC #23224), and B. cereus (ATCC #14579) were obtained from the American Type Culture Collection, Rockville, MD. E. coli RC709 host strains containing large-molecular-weight plasmids for use as standards on agarose gel electrophoresis were supplied by Ester Lederberg, Plasmid Reference Center, Stanford University School of Medicine, CA.

In vitro elimination of plasmids. An exponentially growing culture of B. anthracis Sterne was diluted to about 10^3 CFU/ml in trypticase soy broth (TSB, Difco), pre-warmed to 37°C, containing various concentrations of novobiocin. Cultures were incubated at 37°C for 18 h. Growth was measured turbidimetrically with a Klett-Summerson colorimeter with a red filter (transmission 640 to 700 nm). Cultures which contained a concentration of novobiocin just sub-inhibitory to cell growth (<0.7 µg/ml novobiocin) were plated. Putatively cured colonies were lysed and screened for plasmid content by agarose gel electrophoresis. Strains that had lost the large-molecular-weight plasmid pXO1 concomitantly lost the ability to produce toxin, as determined by a rat lethality assay and by an immunodiffusion assay. Serial passage at elevated temperature (43°C) was also effective in curing Sterne of the toxin-encoding plasmid pXO1.

Rat lethality assay. Strains GK8N and GK40N, generated by curing B. anthracis Sterne of the pXO1 plasmid, and nontreated, plasmid containing B. anthracis Sterne were grown to late-log phase in synthetic R-medium (24). Following centrifugation, culture supernatants were sterilized by filtration through low-protein-binding membranes (Gelman Sciences, Inc.). Culture filtrates were injected into the dorsal penile vein of 300-g Fisher 344 albino rats (2) which were monitored for time-to-death (6).

Toxin immunodiffusion assay. Assay plates were made for the detection of individual toxin-producing colonies by mixing 6 ml of double strength synthetic R-medium with 8 ml of 3% melted agarose at 56°C, and 2 ml of sterile serum from a goat immunized with a veterinary Sterne spore vaccine (Anvax, Burroughs Wellcome Co.). Sterile toothpicks were used to transfer putatively cured B. anthracis colonies to the assay plates. Following 24-h incubation in a 5% CO_2 incubator at 37°C, colonies were examined for the presence or absence of halos of toxin-antitoxin precipitate.

Detection of B. anthracis plasmid DNA. Isolation of plasmid DNA from cell lysates was performed by a modification of the procedure described by Kado and Li[illegible] (12). Strains of B. anthracis were grown in 10 ml TSB to mid-logarithmic phase and harvested by centrifugation. Cells were suspended in 1.0 ml of E buffer (40 mM Tris acetate, 2 mM disodium ethylenediamine tetra acetic acid [EDTA], adjusted to pH 7.9 with glacial acetic acid). Two ml of lysing solution (3% sodium dodecyl sulfate [SDS], 50 mM Tris adjusted to pH 12.6 with NaOH) was added and the sample was gently mixed by inverting the tube several times. The lysate was then heated at 60 to 65°C in a water bath

for 30 min. Lysates were deproteinized with a phenol extraction by adding 6.0 ml of freshly distilled phenol and chloroform (1:1, v/v). The tube was gently mixed by inverting several times and then was centrifuged at 10,000 x g for 15 min at 4°C to separate the phases. The clear aqueous phase was collected with a blunt-end pipette to avoid shearing and analyzed directly by agarose gel electrophoresis.

Agarose gel electrophoresis of plasmid DNA. Aliquots (20-50 µl) of phenol-chloroform-extracted lysate, containing approximately 1 µg of plasmid DNA, were mixed with 20 µl of tracking dye solution (0.7% bromophenol blue, 7% SDS, and 16.5% glycerol in water). Electrophoresis was carried out in 0.7% w/v) agarose gel (Seakem, Marine Colloids, Inc., Portland, Maine) with Tris-borate buffer (89 mM Tris base, 2.5 mM disodium EDTA, and 8.9 mM boric acid, pH 8.3). Samples were electrophoresed at 35 V (constant voltage) at 10°C for 20 h on a 100 X 140 X 3.0 mm vertical slab gel apparatus (model 220, Bio-Rad Laboratories, Richmond, CA). This gave better resolution than running the gel at a higher voltage for a shorter time. Gels were stained with ethidium bromide and photographed as previously described (16). Reference plasmids of known molecular weights were run on all agarose gels to compare with the relative mobilities of *B. anthracis* plasmid DNA bands.

Qualitative test for photoreactivation. Sterile, cotton-tipped swabs were used to make streaks of mid-log-phase cultures of *B. anthracis* and *E. coli* controls on 150 x 25 mm trypticase soy agar plates. Sections of the plates were UV-irradiated at a flux of 0.9 W/m^2 (equivalent to 90 $\mu W/cm^2$ or 0.9 $J\ m^{-2}\ s^{-1}$) to give a cumulative UV exposure of 0, 10, 20, and 30 s to

various portions of the streaks. Duplicate plates were prepared, and one set of plates was exposed to photoreactivating light for 1 h and the other set of plates was kept in the dark.

Quantitative test for photoreactivation and relative UV sensitivity. Cells were grown to mid-exponential phase in TSB (Difco) at 37°C on a rotary shaker. Cells were collected by centrifugation and resuspended in 0.85% saline to 110 Klett units (equivalent to an O.D. of 0.22; approximately 3 x 10^7 CFU/ml for B. anthracis). Ten-ml aliquots were UV-irradiated in open glass petri plates for various times with a General Electric, 15 W, G15T8 germicidal lamp, which emits mainly at 254 nm. The flux was 0.9 W/m^2 as measured with a shortwave UV meter (model No. J-225; Ultra-Violet Products Inc. San Gabriel, CA). The plates were manually agitated during UV exposure to ensure uniform irradiation. Unwanted photoreactivation was avoided by working in a darkroom with an orange photographic safety light. Half of each UV-irradiated cell suspension was kept in the dark. The other half was removed and light-exposed for 60 min by placing the cells in a glass-covered petri plate 12 cm under two Westinghouse, 15 W, F15T8, black-light-blue (BLB) fluorescent lamps with a peak output of 354 nm (11). Plates were covered with glass to filter out the short-wavelength UV component from the BLB lamps. Viable cell counts were determined by plating 0.1 ml aliquots of the appropriate dilutions on trypticase soy agar plates and incubating the plates overnight at 37°C. The surviving factions were plotted as a function of the UV dose.

B. anthracis spore preparation. Overnight TSB cultures of B. anthracis were diluted to about 10^7 CFU/ml in phosphate-buffered saline, and 0.1 ml aliquots were used to inoculate blood agar plates. After incubating at 37°C in a CO_2 incubator for 3 to 4 days, the spores were harvested by washing the blood agar plates with 5 ml/plate sterile, distilled water. Spores were washed once with sterile, distilled water, heat-shocked at 60°C for 30 min to kill vegetative cells, pelleted at 20,000 x g for 20 min, resuspended in an equal volume of 1% phenolized gel-phosphate and stored at 4°C. Spores were diluted to approximately 2 x 10^7 spores/ml in 0.85% saline and tested for UV sensitivity and photoreactivation as described above.

RESULTS

Production and testing of plasmid-free isolates. All toxin-producing strains of B. anthracis tested contained the pXO1 plasmid, and all encapsulated strains contained an additional large plasmid, pXO2, as previously described (1, 33). Growing toxin-producing strains of B. anthracis in the presence of sub-inhibitory concentrations of novobiocin (0.7 μg/ml) or by serial passage at an elevated temperature (43°C) resulted in plasmid-free isolates. Sub-inhibitory concentrations of acridine orange (7.5 μg/ml) and ethidium bromide (0.6 μg/ml) were also used and found to be less effective curing agents.

Intravenous injections of 2 ml of culture filtrate from the nontreated Sterne strain, which contains the pXO1 plasmid, consistently killed Fisher 344 albino rats, with an average time to death of 60 min. Intravenous injections of as much as 8 ml of culture filtrate from Sterne isolates which were cured of pXO1 did not kill rats. By using the toxin immunodiffusion assay, halos of toxin-antitoxin precipitate were observed surrounding plasmid-containing parent colonies of B. anthracis, while halos were not seen around colonies of cells which had been cured of pXO1.

When fifteen different virulent strains of B. anthracis were grown on nutrient agar supplemented with 0.7% $NaHCO_3$ in 5% CO_2, each strain produced predominately smooth mucoid colonies, indicating capsule production (20). Spontaneous, rough, non-encapsulated colonies were also observed at low frequency with each strain. When these cultures were incubated for several days, smooth colonies often developed rough outgrowths which contained

non-encapsulated variants, as previously observed (19, 32). When rough variants from several of these virulent strains were examined for plasmid content, the pXO2 plasmid was missing in each case. This is consistent with recent reports that the pXO2 plasmid mediates capsule production in B. anthracis (5, 33).

Qualitative test for photoreactivation. A rapid, qualitative test for the presence or absence of photoreactivation was used to screen strains of B. anthracis. Figure 1 shows the results of a typical qualitative test for four strains of B. anthracis. Cells were streaked on duplicate TSA plates and exposed to various doses of UV irradiation. Plate A (Fig. 1) was then incubated in the dark while Plate B was exposed to visible light. The increased growth of cells in the UV-irradiated streaks exposed to visible light, over that seen for cells in the UV-irradiated streaks kept in the dark, is indicative of repair of UV-induced DNA damage by photoreactivation.

Quantitative test for photoreactivation. UV dose-survival curves of log-phase cells of Vollum 1B (pXO1, pXO2), Sterne (pXO1), and GK8N and GK40N, which lack both pXO1 and pXO2, were compared. As shown in Fig. 2, these virulent and avirulent strains all had similar UV sensitivities when kept in the dark after UV irradiation. Bacillus cereus, E. coli B/r, the excision-repair-deficient strain E. coli B_{s-1}, and Sterne spores were included for comparison. Figure 3 is a graphic comparison of the relative sensitivities of B. anthracis Sterne spores and Sterne vegetative cells. It also clearly shows the lack of photoreactivation in B. anthracis spores. Results of experiments to determine the increase in survival resulting from photoreactivation in

strains of B. anthracis that contain both pXO1 and pXO2 (Vollum 1B), only pXO1 (Sterne), and neither plasmid (GK8N and GK40N) are illustrated in Fig. 4. All strains of B. anthracis tested showed significant photoreactivation, which was independent of the plasmid profile.

Discussion

Conventional techniques that use Brij-58 and Triton X-100 to prepare lysates for the isolation of small plasmids from *E. coli*, were found to be ineffective for *B. anthracis*. By using a slightly modified version of the method of Kado and Liu (12), I confirmed by agarose gel electrophoresis that all toxin-producing strains of *B. anthracis* tested contained the pXO1 plasmid and all encapsulated strains contained the pXO2 plasmid (data not shown).

A number of bacterial plasmids have been described which alter the sensitivity of the host towards UV light. Some plasmids, such as pKM101, and its parent, R46 (21, 36), confer on the host cells reduced susceptibility to killing by UV irradiation (UV protection). Other plasmids render their host more sensitive to UV, and other plasmids have no effect on UV sensitivity. Some plasmids are known to carry genes which code for repair enzymes (15). When strains of *E. coli*, lysogenic for a transducing phage carrying the *phr* gene, are induced, they can produce approximately 2000 times the normal levels of photoreactivating enzyme (30). In this study, the toxin plasmid pXO1 and the capsule plasmid pXO2 were examined for their role in DNA repair in strains of *B. anthracis*. When I compared UV kill curves of strains harboring one or more of these plasmids with UV kill curves of cured strains, it was clear that pXO1 and pXO2 did not increase UV resistance. Since both plasmid-containing and cured strains of *B. anthracis* demonstrate strong photoreactivation, I concluded that the photoreactivating enzyme is not plasmid encoded.

In addition to being resistant to dryness and heat, spores of B. anthracis are much less sensitive to UV radiation than vegetative cells. More than 99% of the vegetative cells of Vollum 1B and Sterne were killed with 20-s UV exposure (0.9 W/m^2) while 25 min of UV exposure was needed to achieve the same amount of killing of Sterne spores. As shown in Fig. 3, there was no difference in survival when UV-irradiated Sterne spores were grown in the dark or exposed to photoreactivating light, indicating the absence of detectable photoenzymatic repair. Stafford and Donnellan (26) reported that spores of B. cereus, B. subtilis, and B. megaterium become more resistant to UV radiation during the initiation of germination, after which the UV sensitivity rapidly approaches the sensitivity of vegetative cells. Romig and Wyss (25) showed that sporulating cultures of B. cereus lose their photoreactivability at the same time that UV resistance increases. Changes in UV sensitivity due to germination were avoided in this study by storing B. anthracis spores in 1% phenolized gel-phosphate and UV-irradiating the spore suspensions in 0.85% saline. The predominant UV photoproduct in spores of Bacillus species has been identified as 5-thyminyl-5, 6-dihydrothymine (22). Unlike the UV-induced cyclobutyl-pyrimidine dimers (cyclo-butadipyrimidines) formed in the DNA of vegetative cells, the spore photoproduct is not monomerized by photoreactivation. However, B. subtilis spores do possess an enzymatic dark repair system which can monomerize the spore photoproduct in situ during germination (8).

Although UV resistance is primarily a function of the repair efficiency of the organism, Nasim and James (23) have suggested that pigments can affect the level of UV resistance by acting as an energy sink. In one case (9), pigment production increased photoreactivation, possibly by supplying energy indirectly to the photoreactivating enzyme. In chemically defined liquid medium containing iron salts, wild-type strains of B. anthracis produce a soluble pink-to-purple pigment which has been identified as protocatechuic acid (3,4-dihydroxybenzoic acid) (3). The effect of this pigment production on UV resistance and photoreactivation has not been examined.

Solar radiation contains a short-wavelength component that forms potentially mutagenic and lethal photoproducts in DNA (18). Wavelengths down to 300 nm are commonly observed. Occasionally, in very clear atmospheres, 280 nm is seen (27). Intensity varies with solar angle, altitude, cloud cover, and the concentration of ozone (4). Even wavelengths in the near-UV range (320 to 400 nm) can damage DNA, as shown in experiments with repair-deficient E. coli recA$^-$ uvrA$^-$ (37). Ultraviolet tolerance and photo-induced repair would be advantageous for B. anthracis which is exposed to both UV and photoreactivating wavelengths in sunlight when growing in its natural soil environment. This reasoning is consistant with the findings presented in this paper that vegetative cells of B. anthracis are capable of DNA repair by photoreactivation.

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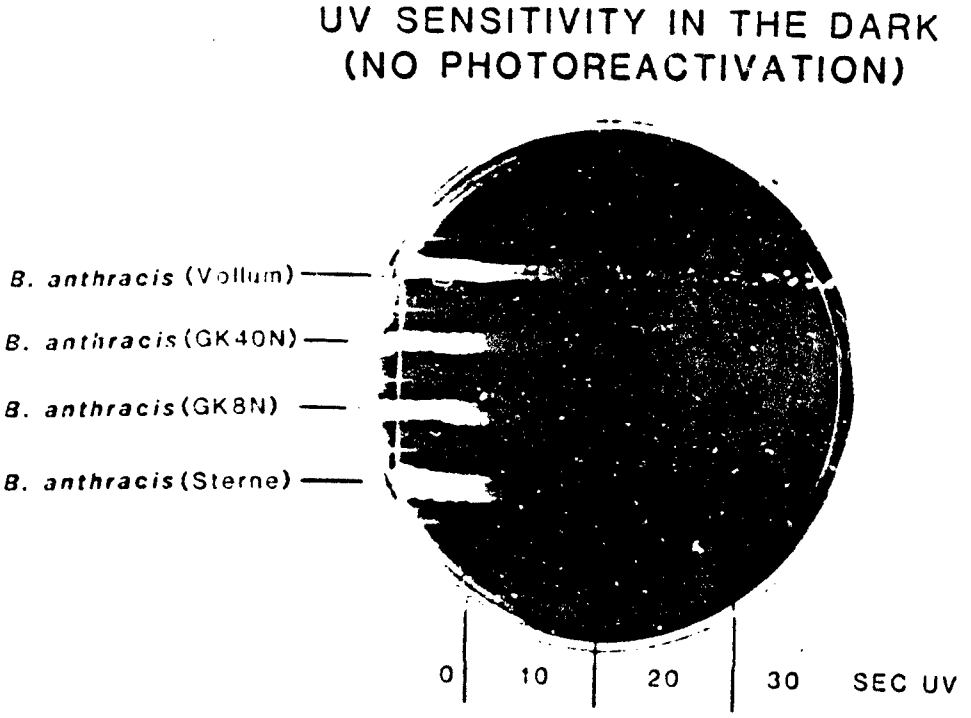
A.



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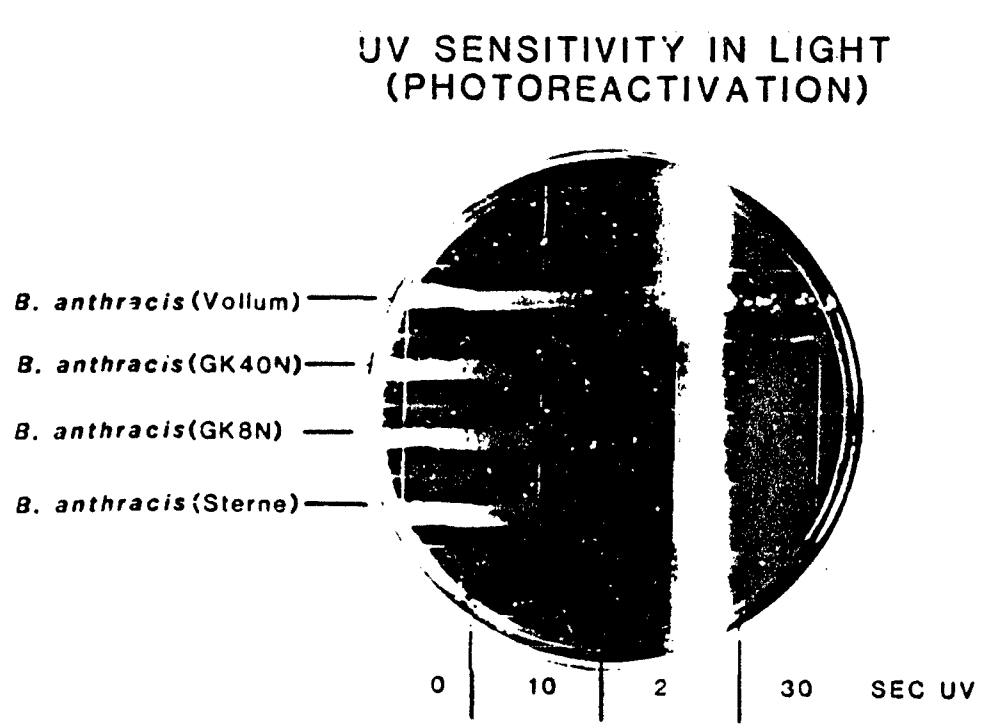

FIG. 1. Log-phase broth cultures of various B. anthracis strains were streaked on trypticase soy agar plates and exposed to UV-irradiation for 0, 10, 20, and 30 s. Plate A was kept in the dark while plate B was exposed to visible light for 60 min. The plates were then incubated at 37°C for 18 h.

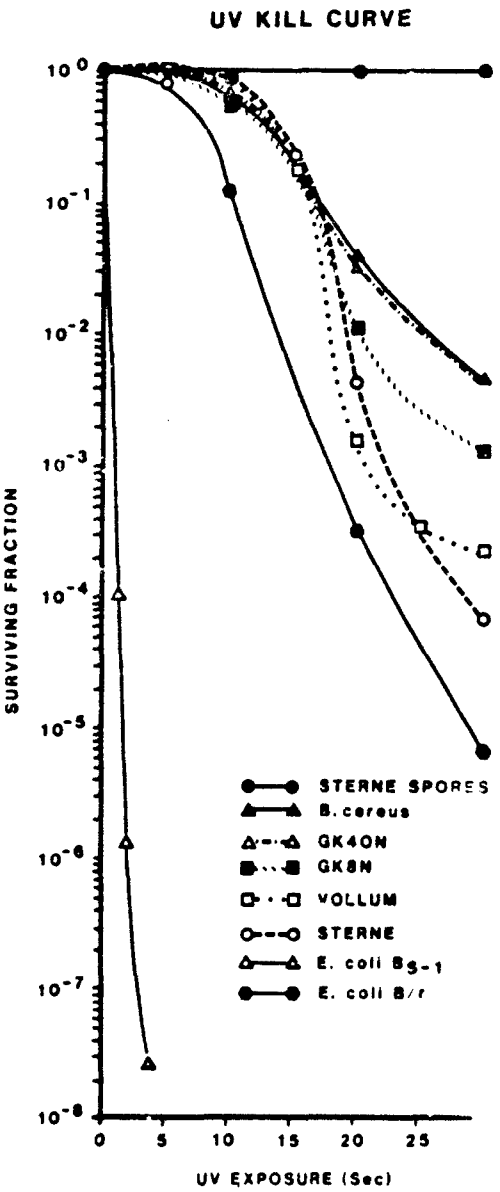

FIG. 2. Comparison of UV sensitivity among strains of B. anthracis, Sterne spores, and controls.

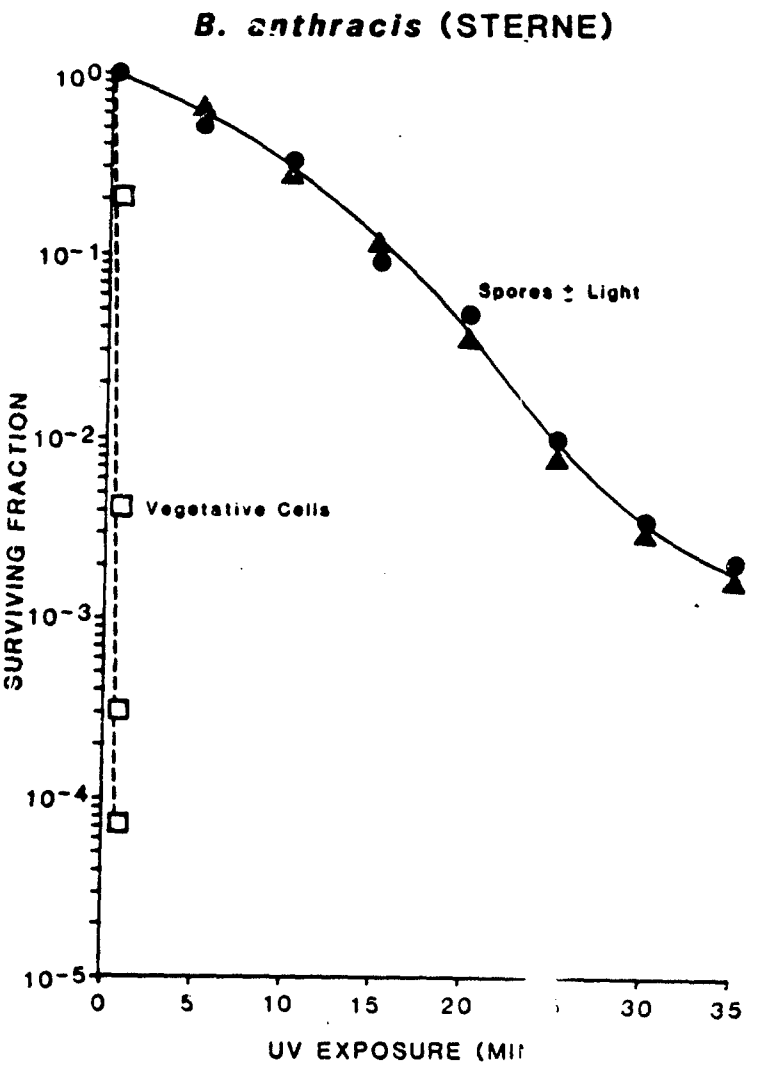

FIG. 3. UV dose-survival curves for B. anthracis Sterne spores with photoreactivation light (●) and in the dark (▲), and Sterne vegetative cells in the dark (□).

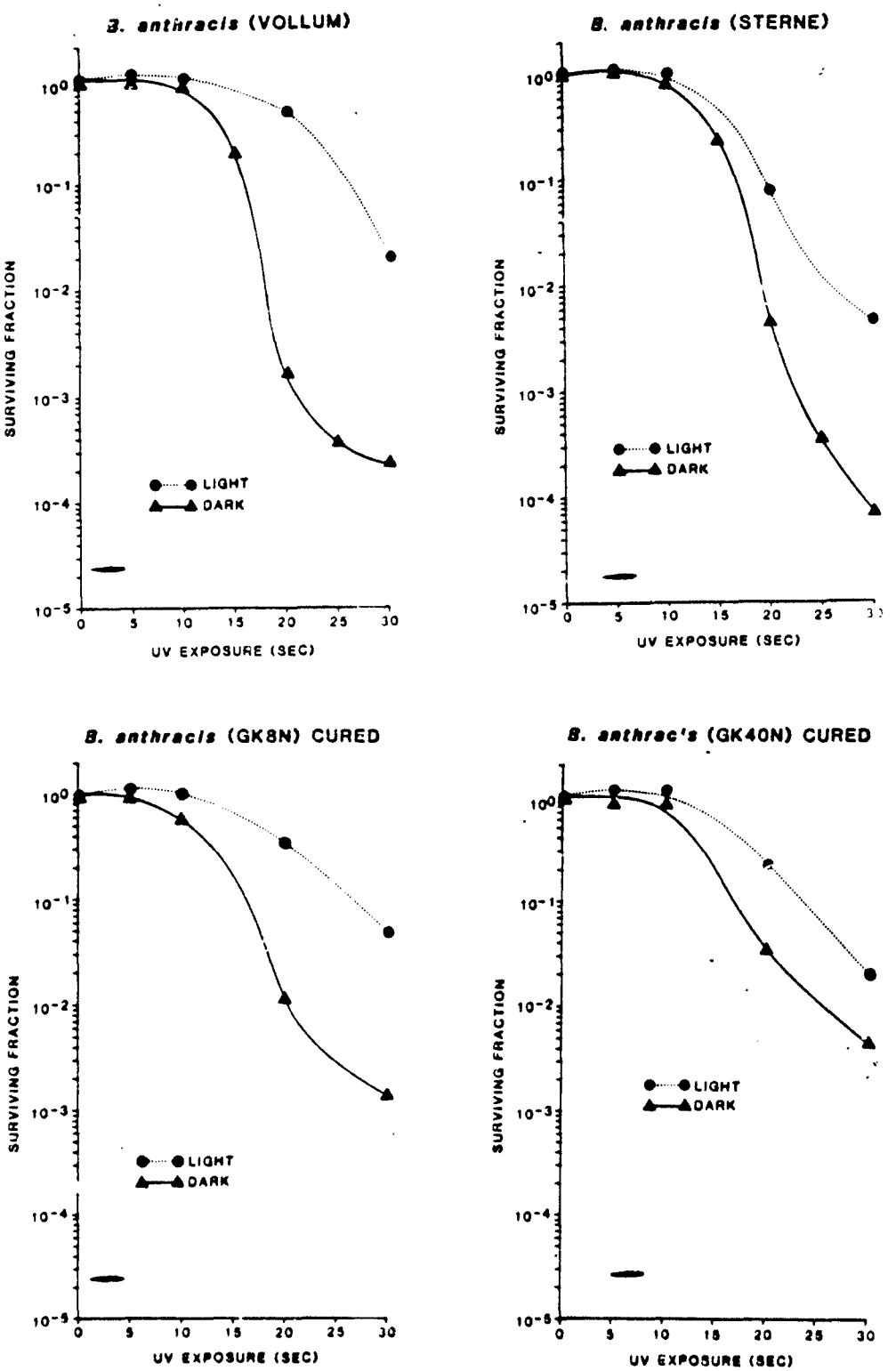

FIG. 4. UV-inactivation and photoreactivation of four strains of B. anthracis. Survival with photoreactivation light (●) and in the dark (▲) as a function of UV dose.